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Hollow silica microspheres as robust immobilization carriers

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ABSTRACT

Hollow silica microspheres provide an ideal solid support for enzyme immobilization. We tested one of the newest development, namely MATSPHERES®, a silica openwork material as a carrier for the covalent immobilization of enzymes used to synthesize bioactive compounds. Two model enzymes – ethylbenzene dehydrogenase and EL070 lipase – were considered. They belong to two different enzyme classes and catalyse reactions taking place in various environments (aqueous and non-aqueous, aerobic and anaerobic). The enzymes were immobilized by covalent bonds (*via* divinyl sulfone and glutaraldehyde) on new silica material. Effectiveness of immobilization processes on the spheres grafted with amine groups and on the analogues without functionalization was determined for both enzymes. Microspheres were characterized morphologically and also their mechanical stability was examined during exposure to varying physical conditions. It was shown that MATSPHERES® due to their openwork structure and relative stability under batch and flow conditions can be a competitive SBA support for enzyme immobilization and production of bioactive compounds.

1. Introduction

In the era of frightening industrial growth, it became necessary to look for solutions that are less harmful to the environment, in either the production of new goods or waste management. Therefore, acquisition of new biologically active compounds with the aid of biocatalysis is gaining more and more popularity. *Green Chemistry* employing enzymes in compound processing offers a wide range of advantages over traditional ways: reduction in energy and water consumption, minimizing the use of hazardous, toxic chemicals and solvents as well as decrease in waste emission and pollution [1,2]. Enzymes used in biocatalysis allow to obtain a wide range of compounds, including those applied in food and pharmaceutical industries, in an extremely selective manner. Moreover, recent developments in biocatalysis open new routes for utilisation of compounds originating from various resources, also those constituting as a waste. Enzymes can be applied either in the synthesis of new drugs or intermediate products exhibiting desired properties. However, despite huge advantages, enzymes, as products of living organisms, have small tolerance to a range of physicochemical and biological parameters (i.e. temperature, pH, solvents and proteolytic enzymes etc.) [3]. Thus, studies elucidating improvements of the enzyme efficiency, reaction optimisation routes or a search for the most optimal reaction conditions are essential. To overcome the problem of their vulnerability, enzymes may be immobilized on a range of different supports. Immobilization increases their stability and activity, which has a significant impact on improving the overall efficiency of catalysed reaction [4]. The conversion rate for immobilized enzymes is also found to be higher compared to their free forms (only in case of lipases) [5] and organic solvent resistance is significantly increased [6]. Moreover, immobilization greatly facilitates the re-use of the catalysts [7–9].

Due to a huge number of potential applications of enzyme immobilization there is a growing interest in searching for and testing new supports which meet the requirements of specific enzymes or reactions conducted by them [10]. For example, silica based microspheres constitute a new interesting group of enzymes attaching carriers. Silica capacities to bind an enzyme on its surface were already widely studied [11–13]. Most importantly, silica as a support is chemically versatile; it offers great variation of its chemical surface modification enabling different approaches for covalent binding of a given enzyme [14]. Further, recent developments in material design enable creation of 3D spheres of different sizes and architecture [15–17]. Openwork spheres for example not only provide greater surface for immobilisation of an

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enzyme when compared to simple granules but also support reduction on operating pressure in flow bioreactors enabling higher flows for particular applications. In the presented work we have focused on one of such new developments – on MATSPHERES® – hollow silica microsphere of sophisticated 3D architecture for immobilisation of two distinct enzymes – a dehydrogenase and a lipase, which perform their catalytic reactions in two different reaction media – aqueous and non-aqueous.

Both enzymes selected for this study are of pharmaceutical and chemical importance. Both are used to synthesize bioactive compounds with a variety of applications. The first enzyme considered in this study is an ethylbenzene dehydrogenase (EbDH) belonging to DMSO reductase family [18]. It was firstly isolated from *Aromatoleum aromaticum* (EbN1 *Azoarcus* sp.) where it is responsible for anaerobic ethylbenzene mineralization and converts wide range of substrates into chiral secondary alcohols [14–24], which are considered as Active Pharmaceutical Ingredients (APIs). EbDH is one of the few enzymes conducting synthesis of secondary chiral alcohols by enantioselective hydroxylation of hydrocarbon substrates structurally similar to ethylbenzene. What is especially noteworthy is the fact that out of 34 known EbDH substrates, 21 result exclusively in (*S*) enantiomer [25–27]. For example, natural product of EbDH transformation of ethylenbenzene, that is 1-phenylethanol, is found to be utilized as a synthon for i.e. selective A1 adenosine receptor antagonists synthesis, a stimulating agent of the central nervous system [28,29] or in synthesis of dopamine transporter ligands (DAT) as a novel potential cocaine-abuse therapeutic agent [30]. The other enzyme used in this study is a lipase, commercially available from Eucodis Bioscience GmbH (Austria) under trade name EL070. Lipases (EC 3.1.1.3.) are a subclass of the hydrolases that under physiological conditions play crucial role in hydrolysis of triacylglycerols, while in the absence of water are prone to catalyze reversed reactions (i.e. esterification). It was reported that lipases promote efficient synthesis of sugar fatty acids (SFAEs) [6,31–33]. SFAEs constitute a novel group of non-ionic sugar-based surfactants increasingly used as emulsifiers in pharmaceutical, cosmetic, food and detergent formulae. Biodegradability and biocompatibility, no irritating effect and lack of toxicity of those compounds are favourable features that raise the great interest of their additional broad applications in food and pharmaceutical industries [34]. They were shown to bare antibacterial properties resulting from surfactant interactions with bacterial cell membrane [35] and also they can be potentially implemented as anticancer agents [36,37].

Having the above in mind, we have conducted a systematic study that encompassed characterisation of MATSPHERES® hollow silica microspheres, their application for two types of enzymes immobilisation, which was followed by catalytic testes in both batch and flow modes.

2. Materials and methods

2.1. Materials

Commercial lipase EL070 (EC 3.1.1.3) of prokaryotic origin (highly purified lyophilized powder) was obtained from Eucodis Bioscience (Austria). Ethylbenzene dehydrogenase (EbDH) (EC 1.17.99.2) was obtained as described elsewhere in detail [27]. Briefly, *Aromatoleum aromaticum* was cultivated under anaerobic conditions, cells were collected and free cell extract was submitted to column purification (Sepharose DEAE, Hydroxapatite). Purity of enzyme was assayed by SDS-PAGE (data not shown). Immobilisation carriers were provided by Materium Innovations (Canada) and referred to in this manuscript as MATSPHERES®–OH (non-modified) and MATSPHERES®–NH_2 (modified as described below). SBA-15-ultra-2 carriers were obtained as described in [38] and referred to in this manuscript as SBA-OH (non-modified) and SBA-NH_2 (modified as described below). Other chemicals were of reagent grade and purchased from Merck (Poland) and VWR (Poland).

2.2. Physical characterisation of silica spheres

The size of MATSPHERES® was confirmed by light microscopy observations (Nikon ECLIPSE E200; 7 photos, magnification 400x, sample size 136 objects) and analysed with ImageJ 1.49 v software. The shape of the microspheres after short activity assays and long-term reactions was also examined with an optical microscope in order to evaluate the influence of reaction conditions on the structural integrity of the carriers. Similarly, the effect of freezing immobilization support ($-20\,^{\circ}C$, 2 weeks period) was also determined by microscopic observations. The effect of the flow rate was studied with Agilent 1100 Series binary pump by varying the flow up to 5 ml min^{-1} for PrepRPC FPLC column (Pharmacia Biotech, Sweden) packed with 0.5 ml of each of the supports studied. Specific surface area (S_{BET}), pore volume (V_{tot}) and pore diameter of SBA were obtained as described elsewhere [38]. SBA grain size was determined based on electron microscope measurements (JEOL JSM-7500F). MATSPHERES® characteristics were provided by the supplier (including S_{BET}, V_{tot}, pore diameter and density of functional groups). The interaction of spheres with of media components was verified by appropriate HPLC measurement as described below. Each component (i.e. glucose, methyl nonanoate, 1- (*S*)-phenylethanol, $K_3[Fe(CN)_6]$ in respective reaction media was incubated over appropriate time which depended on the assay performed and its concentration was analysed.

2.3. Enzyme immobilization

Enzymes immobilization was carried out accordingly to scheme at Fig. 1. Immobilization of each enzyme (EbDH or lipase) was conducted with 0.5 ml of each carrier (MATSPHERES®–OH and MATSPHERES®–NH_2 silica microspheres, SBA-OH or SBA-NH_2).

2.3.1. EbDH immobilization

The carriers (0.5 ml) were rinsed twice with distilled water and then with 20 ml of buffer appropriate to the type of surface activation, i.e. 1 M Na_2CO_3 (pH 11) for silica-OH carriers, 0.1 M KH_2PO_4/Na_2HPO_4 (pH 7.0) for silica-NH_2 carriers. Next the surface of the carriers were activated according to the following procedures: i) silica-OH carriers were slowly agitated with 0.5 ml of 3% divinyl sulfone (DVS) in 1 M Na_2CO_3 for 1 h at room temperature using laboratory orbital shaker. ii) silica-NH_2 carriers were stirred with 0.5 ml 2.5% glutaraldehyde (GA) in 0.1 M KH_2PO_4/Na_2HPO_4 for 1 h at room temperature. After activation with DVS and GA the supports were rinsed with distilled water and subsequently with 0.1 M Na_2HPO_4 (pH 8.0) or 0.1 M KH2PO4/Na_2HPO_4 (pH 7.0), respectively. Moreover, in order to protect the enzyme during immobilization all buffers were supplemented with 3 mM $K_3[Fe(CN)_6]$. For each type of the carrier 0.5 ml of the purified EbDH (3.67 mg/mL, activity: 27.18 mU/mL) was added and gently stirred for 1 h at room temperature after which it was cooled to 4 °C and left at this temperature for 3 h. The excess of unbound protein was filtered off and carriers were washed with 0.1 M KH_2PO_4/Na_2HPO_4 (pH 7.0) to remove the excess of unbound protein. All eluates were collected and analyzed for the presence of the unbound protein. Finally, in order to endcap the free active groups of the carrier, the supports were submerged in 0.5 M Tris/HCl buffer pH 7.5 for 3 h at 4 °C. At this stage immobilization of EbDH was completed and each carrier-EbDH was left at 4 °C.

2.3.2. Lipase immobilization

For each ml of carrier 100 mg of lipase was prepared. 10 mg of lipase was weighed for control sample used in protein activity assay. Lipase was dissolved in buffer solutions pH = 8.0 and pH = 7.0, respectively for silica-OH and silica-NH_2. Lipase concentration in each of

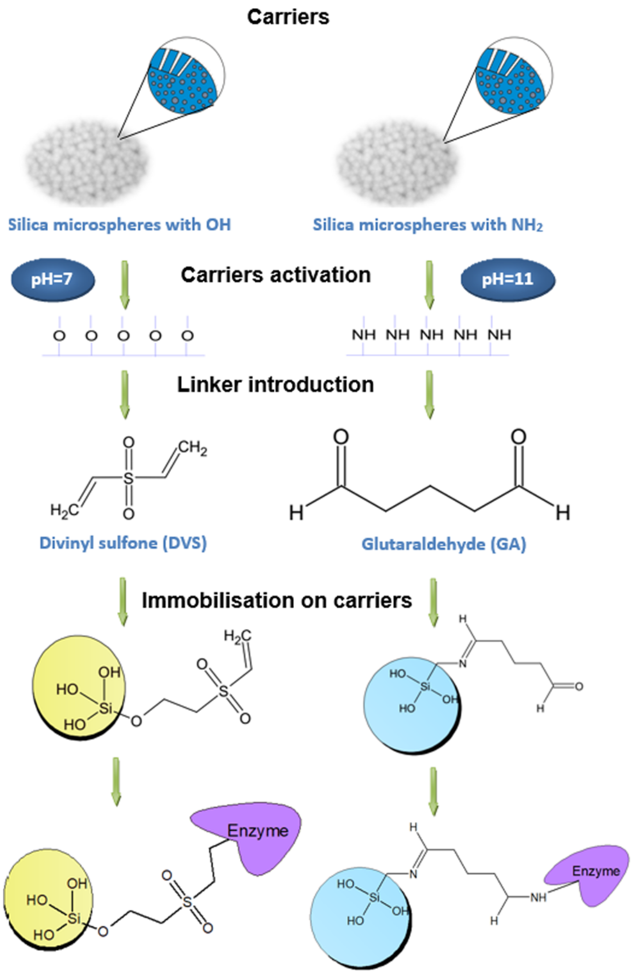

Fig. 1. Schematic representation of immobilization procedures used in this work.

the buffers was 50 mg ml^{-1} or 30.2 mg ml^{-1} for MATSPHERES® or SBA, respectively. The carriers (0.25 ml) were rinsed twice with 0.5 ml of the buffer appropriate to the type of surface activation, i.e. 1 M Na_2CO_3 (pH 11.0) for silica-OH carriers, 0.1 M KH_2PO4/Na_2HPO_4 (pH 7.0) for silica-NH_2 carriers. Next, in order to activate the carriers surface, the following procedures were conducted: i) silica-OH carriers were slowly agitated with 0.5 ml of 3% DVS in 1 M Na_2CO_3 for 1 h at room temperature using laboratory orbital shaker; ii) silica-NH_2 carriers were stirred with 0.5 ml 2.5% GA in 0.1 M KH_2PO_4/Na_2HPO_4 for 1 h at room temperature. After activation, each carrier was rinsed six times with deionized water in order to remove the excess of the linker. Then, each was suspended in a buffer solution (0.1 M Na_2HPO_4, pH = 8.0) and stored for 3 h at 4 °C. After that time, silica-OH and silica-NH_2 carriers were rinsed twice with 0.1 M Na_2HPO_4 (pH 8.0) or 0.1 M KH_2PO4/Na_2HPO_4 (pH 7.0), respectively. For each type of the carrier 0.5 ml of the lipase solution was added and gently stirred for 1 h at room temperature after which it was cooled to 4 °C and left at this temperature overnight (Note: enzyme was dissolved/suspended in 0.1 M Na_2HPO_4 (pH 8.0) or 0.1 M KH_2PO4/Na_2HPO_4 (pH 7.0), respectively for silica-OH and silica-NH_2 carriers). The excess of unbound protein was filtered off and carriers were rinsed four times with 0.1 M KH_2PO_4/Na_2HPO_4 (pH 7.0). All eluates were collected and analyzed for the presence of the unbound protein. Finally, in order to endcap the free active groups of the carrier, the supports were submerged in 0.5 M Tris/HCl buffer pH 7.5 and gently stirred at room temperature for 1 h. Next, each carrier was rinsed with deionized water and freeze-dried overnight.

The success of immobilization process for each enzyme was described by "enzyme loading" and "retained activity" parameters as described in Eqs. (1) and (2).

$$\textbf{Enzyme Loading}(\%) = \frac{\text{immobilized protein [mg]}}{\text{initial amount of protein [mg]}} \times 100\% \tag{1}$$

$$\textbf{Retained Activity}\ (\%) = \frac{\text{observed activity [U]}}{\text{initial activity [U]}} \times 100\% \tag{2}$$

2.4. *Reagents sorption studies*

Reagents used for EbDH: 0.25 ml (66 mg MATSPHERES®–OH and 63 mg MATSPHERES® –NH_2) of a carrier was suspended in 10 ml of 100 mM MES buffer (pH 5.5) containing 1.55 mM 1-(*S*)-phenylethanol (PhEtOH) or 1 mM electron acceptor potassium ferricyanide (III) $K_3[Fe(CN)_6]$. The mixture was continuously shaken (temp. 30 °C, 250 rpm) for 3 days and 0.25 ml samples were removed periodically. Samples were centrifuged (13,000 rpm) and submitted for quantitative HPLC and UV–vis analysis. Reagents used for EL070: 0.25 ml (66 mg MATSPHERES®–OH and 63 mg MATSPHERES®–NH_2) of a carrier was suspended in 10 ml of reaction medium (80% v/v of 2-methyl-2-butanol and 20% v/v DMSO) containing 4 mg ml^{-1} glucose. The mixture was continuously shaken (temp. 30 °C, 250 rpm) for 3 days and 0.25 ml samples were removed. Samples were centrifuged (13,000 rpm) and submitted for quantitative HPLC-MS analysis.

2.5. *Determination of protein concentration*

The amount of protein in the supernatant was determined by Bradford method using Bovine Serum Albumin as a standard [39]. The amount of bound protein was determined indirectly by the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein in the filtrates. Measurement were performed using spectrophotometer microplate reader (Epoch, BioTek, Instruments, Inc.) controlled with a Gen5 Data Analysis software interface. Immobilization efficiency was calculated by subtracting the concentration of the tested supernatants from the initial concentration of the enzyme solution.

2.6. *Determination of EbDH activity*

Enzymatic tests were carried out in three modes: batch, fed-batch and continuous flow, under aerobic and anaerobic conditions. 0.5 ml of homogenous or immobilized EbDH enzyme (carrier suspension) was added into 10 ml (batch mode, aerobic conditions) or 20 ml (fed-batch mode, anaerobic conditions) of 0.1 M Tris/HCl buffer (pH 7.5) with 6 mM $K_3[Fe(CN)_6]$. Reaction buffer was preheated to 30 °C. The reactions were initiated by addition of ethylbenzene stock solution in *tert*-butyl alcohol (C = 1.5 mM). The initial activity was determined under aerobic conditions. An EbDH activity unit (U) was defined as the amount of enzyme liberating 1 μmol of 1-(*S*)-phenylethanol per minute under the assay conditions. Activity was calculated in U/mL of settled immobilized preparation or in U/mg of immobilized enzyme. Degree of reaction conversion was calculated as the ratio of the product concentration at the end of the reaction to the initial substrate concentration. Long term fed-batch enzymatic experiments were carried out under anaerobic conditions in order to extend the enzyme operation stability [27]. The substrate and re-oxidant were supplemented whenever depletion was observed by HPLC or UV–Vis (420 nm), respectively. HPLC determination of substrate and product was conducted as described elsewhere [27]. Immobilized EbDH was tested in the flow regime under anaerobic conditions by measuring the concentration of the product obtained at a specific flow rate. The range of flow rates varied from 0.05 to 1.5 ml min^{-1} and reactions were carried out in the same conditions as described above. Based on these measurements, the

Table 1
Physical analysis of immobilization supports.

Carrier	Particle shape and size	S_{BET} [$m^2 g^{-1}$]	V_{tot} [$cm^3 g^{-1}$]	Amine group density [$mmol\,g^{-1}$]	Vinyl group density [$mmol\,g^{-1}$]	Pore diameter [Å]	Mass of 0.5 ml of carrier [mg]
SBA-OH	Cube, 190 ± 49 nm by 580 ± 144 nm	692	1.95	—	—	140	52
SBA-NH_2		490	1.62	1.0	—	140	74
MATSPHERES® –OH	Sphere, 30 ± 10 µm	212	1.52	—	—	150–300	132
MATSPHERES® –NH_2		189	1.41	0.5	0.5	150–300	125

dependence of substrate conversion to residence time was determined. Residence time was calculated as the ratio of the bed volume (including the volume of the carrier being compared) to the flow rate. Having the above in mind, the most optimum flow rate was selected.

2.7. Determination of free and immobilised lipases enzymatic activity towards tributyrin

Enzymatic activity was determined by employing a modified protocols [40–42]. Briefly, tributyrin solution was prepared in HEPES buffer (pH 7.2) with addition of 25 µl–50 µl of Triton X per 20 ml of the reaction mixture. Lipase *Thermomyces lanuginosus* (TL-IM, Science Technics Sdn Bhd; Malaysia), *Candida antarctica* lipase B (CalB, Novozym 435; 10,000 PLU/g; Denmark) and EL070 (free and immobilized on different supports) were incubated in the solution with constant stirring (150 rpm) at 55 °C. Samples were withdrawn at specific intervals, diluted 10x with acetonitrile and analysed at Shimadzu Prominence HPLC equipped with UV–Vis detector set to 210 nm and Eclipse XDB-C18 column 4.6 × 100 um Agilent Technologies (depletion of tributyrin, increase of acetic acid signal). An isocratic elution of 15% water and 85% acetonitrile was applied. The injection interval was 4.5 min. Lipase activity unit (U) was defined as the amount of the enzyme that catalyzes the conversion of 1 µmol of tributyrin per minute under the specified conditions of the assay method.

2.8. Determination of EL070 activity in SFAE synthesis

Enzymatic reactions were carried out either in 100% v/v 2-methyl-2-butanol (2M2B) or in 2M2B/DMSO (80:20, v/v). Glucose was supplemented to yield 4 mg ml^{-1}, whereas methyl nonanoate to 11 mg ml^{-1}. 50 mg of molecular sieves (4 Å) was added to withdraw forming water. Reactions were initiated by addition of 12 mg of an enzyme (TL-IM, CalB or EL070 free or in immobilized form). Reactions were conducted at 55 °C either in a batch mode (24 h, rotation in a BINDER thermostat oven MASON Technology) or in a continuous flow mode. PrepRPC FPLC packed column was connected to HPLC pump Applied Separations K-500). Samples were collected at set intervals and were analysed on HPLC-MS system as described below. Lipase activity unit (U) was defined as the amount of the enzyme that catalyzes the conversion of 1 µmol of glucose per minute under the specified conditions of the assay method.

2.9. HPLC measurement

Glucose qualitative analysis was performed by HPLC measurements on Agilent 1290 Infinity System with automatic autosampler and MS Agilent 6460 Triple Quad Detector equipped with Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm). For separation of sugar fatty acid esters (SFAE) the column was eluted at 30 °C at a flow rate of 0.4 ml min^{-1} and developed with gradient elution of mixture of water (A) and methanol (B) given as follows: 0.00 min (95% A/5% B) to 1.00 min (0% A/100% B) to 3.50 min (0% A/100% B) to 3.51 min (95% A/5% B) to 4.50 min (0% A/100% B). The injection interval was 4.5 min. MS Agilent 6460 Triple Quad tandem mass spectrometer with Agilent Jet Stream ESI interface was used in negative ion mode. Nitrogen at a flow rate of 10 L min^{-1} was used as the drying gas and for collision-activated dissociation. Drying gas and sheath gas temperatures were set to 350 °C. Capillary voltage was set to 3500 V, whereas the nozzle voltage was set to 500 V. Glucose was monitored by MRM transition of 179 → 89 m/z in negative ion mode and its depletion was calculated from calibration curve. MassHunter software (Agilent) was used for HPLC-MS system control, data acquisition, and data processing.

3. Results and discussion

3.1. Physicochemical characterisation of immobilisation supports

It is well known that grain size, pore architecture and carrier surface functionalization are very important factors that affect the applicability of a carrier. Therefore, we have performed basic physical analysis of MATSPHERES® and compared them with standard Santa Barbara Amorphous (SBA-15) immobilization carriers (Table 1). Microscopic observations revealed that the MATSPHERES® supports are hollow micrometric spheres, whereas SBA, a commonly used enzyme immobilization support, is a powder consisting of nanometric cubes. Nitrogen adsorption studies showed that the available surface area of the hollow spheres was 2.6 to 3.4 fold lower than these of SBA (for details see supplementary material). There was also lower coverage of the ammonium immobilisation on the spheres. However, the spheres are characterised with up to 2 fold larger pores with greater variety in sizes due to the openwork architecture, which positively influences enzyme immobilization by enabling its penetration and encapsulation in the sphere's core. Mechanistic studies revealed that the hollow spheres are not resistant to mechanical stirring with magnetic bar, however they withstand prolonged rotation (i.e. application in batch type reactors) and elevated pressures obtained by relatively high flows (up to 5 ml min^{-1}) (Fig. 2). In addition, the pressure generated by a column filled with 0.5 ml MATSPHERES® at a flow rate of 3 ml min^{-1} corresponds to the pressure that generates the same volume of SBA-15 at three times lower flow rate (Fig. 2E). Thus, the hollow microspheres outperform the standard SBA supports for applications in flow through reactors. Neither lyophilisation nor cycle of freezing the wet supports at −20 °C and thawing had any effect on the spheres architecture. Finally, storing of MATSPHERES® at −20 °C in aqueous solution for two weeks did not destruct their structure (Fig. 2G). Such stability may be due to perforated structure of this material.

3.2. Carriers' interactions with the reaction components

For the purpose of demonstrating the universality of hollow microspheres in biocatalysis, we have chosen two distinct enzymes of interest for pharmaceutical and food industries that carry out their catalytic reactions in different media, i.e. aqueous and non-aqueous, ethylbenzene dehydrogenase (EbDH) and a lipase (EL070) respectively. These two enzymes greatly differ in the mode of action (Fig. 3) – EbDH

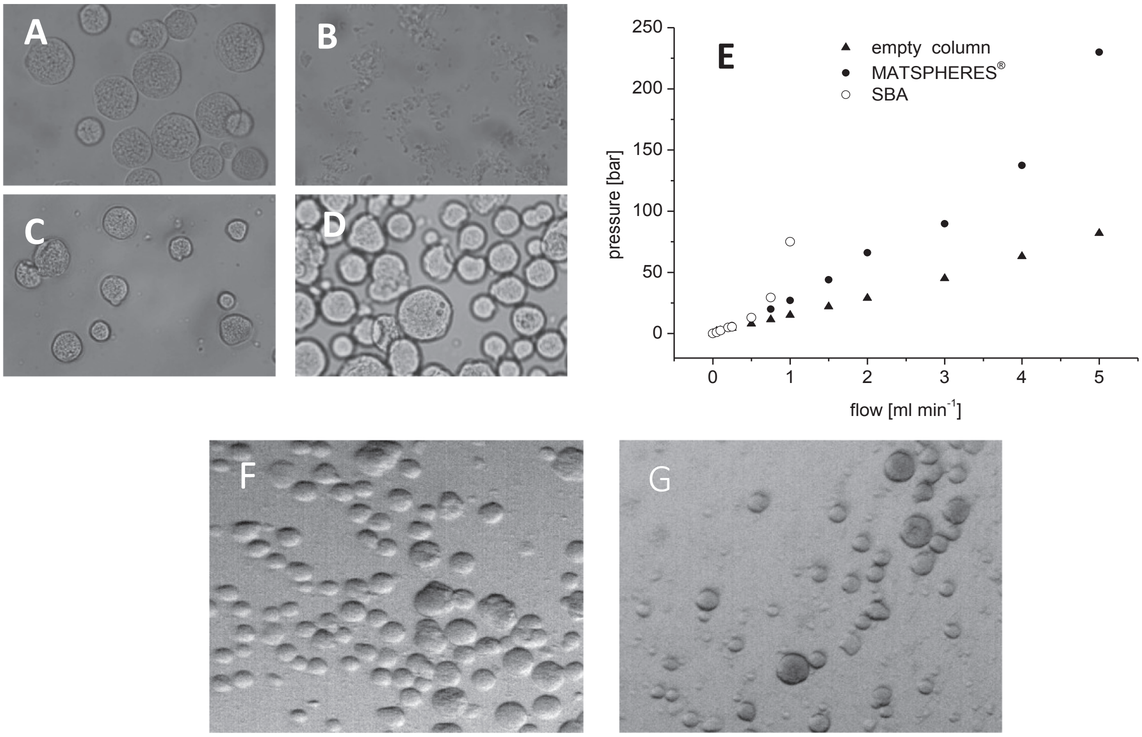

Fig. 2. Mechanistic performance of hollow spheres. Microscopic observations of spheres under different condition (magnification 400 X): (A) unmodified carrier material, (B) material after 24 h stirring in solution with magnetic bar, (C) material after constant rotation for 48 days, (D) material after quadruple flow of solvent at 0.05 ml min^{-1}, (E) pressure as a function of the flow rate generated by the empty column and a column filled with 0.5 ml of silica. Microscopic image of MATSPHERES® before (F) and after (G) 14 days of storing at −20 °C.

converts ethylbenzene in buffered water solution to 1-(*S*)-phenylethanol and requires an artificial electron acceptor ($K_3[Fe(CN)_6]$) for its action *in vitro*, whereas lipase is capable of generating of a novel ester bond in non-aqueous environment without aid of any mediator. Therefore, we have conducted several experiments in order to determine whether the hollow spheres interact with any given component of both reactions. Firstly, we verified whether the hollow spheres display any interaction with both enzymatic substrates under the reaction condition employed in this study. We did not observe any relevant physical nor chemical interplay for any of the tested compounds, with exception to $K_3[Fe(CN)_6]$. When potassium ferricyanide(III) was contacted with MATSPHERES®–OH and SBA-OH in EbDH reaction buffer (1 mM $K_3[Fe(CN)_6]$) remained on the same concentration level over 312 h. However that was not the case for the spheres with NH_2 and vinyl functionalities (MATSPHERES®–NH_2 or SBA-NH_2), where a rapid decrease to depletion was observed (Fig. 4). This can be explained by the $K_3[Fe(CN)_6]$ oxidation reaction of distal –NH_2 groups, and therefore we conclude the MATSPHERES®–NH_2 are less desirable in use for EbDH biocatalysis.

3.3. Enzymes immobilization on the hollow spheres and their enzymatic performance

Enzymatic preparation of ethylbenzene dehydrogenase obtained from a native strain *Aromatoleum aromaticum* EbN1, as well as commercial EL070 lipase powder, were used for immobilization on two types of spheres – non-functionalized (MATSPHERES®–OH) and functionalized with vinyl and amino groups (MATSPHERES®–NH_2). For

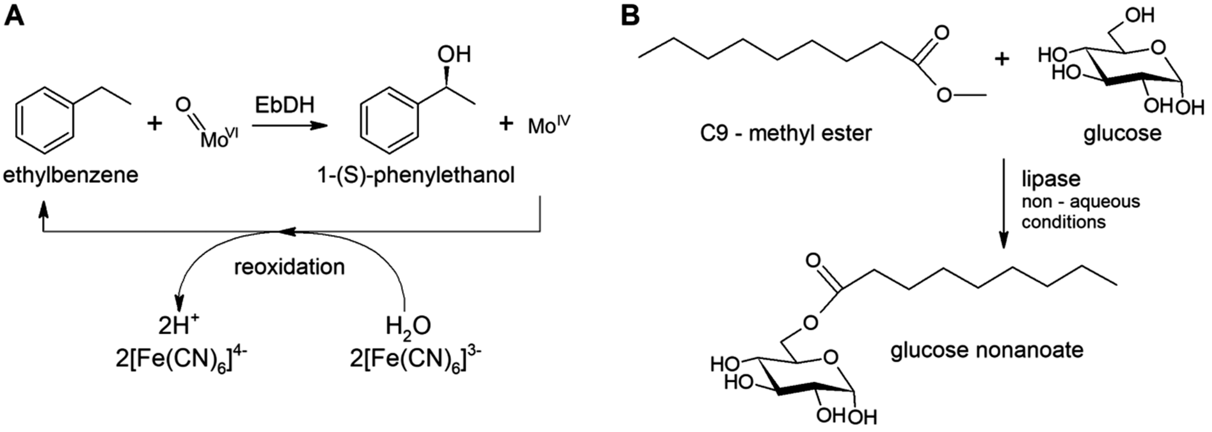

Fig. 3. Reactions performed by enzymes in this study. Panel A – ethylbenzene dehydrogenase converts ethylbenzene to 1-(S)-phenylethanol while transferring an electron to an artificial electron acceptor $K_3[Fe(CN)_6]$. Panel B – lipase EL070 acylates glucose with methyl nonanoate to form sugar ester and a side product methanol.

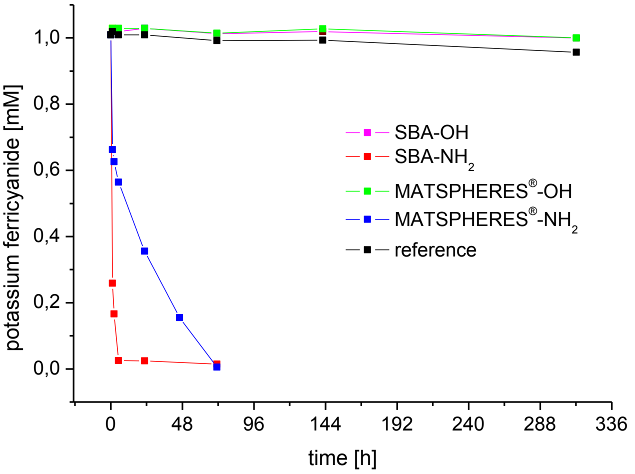

Fig. 4. Interaction of potassium ferricyanide (III) with hollow microspheres. Buffer with electron acceptor without silica stands as a reference.

further comparisons SBA-OH and SBA-NH$_2$ analogous preparations were also made (Table 2).

3.3.1. EbDH

It is known that EbDH class of enzymes is oxygen sensitive [21,43] and therefore these conditions seem to be more favorable for long-term EbDH operation. We have confirmed this phenomenon by performing a batch type reactors under both atmospheres. After immobilization process, there was high protein coverage of both types of spheres, ranging from 88 to 90% or 3.2 – 3.3 mg of bound protein per ml of carrier. Under aerobic conditions and independently of sphere's modification we observed a progressing enzyme inactivation due to the presence of oxygen. Under these conditions, free and immobilized formulation of EbDH were inactivated after about 20 h of continues work [27]. Still, they were able to produce similar amounts of product for all carries (from the lowest for SBA-NH$_2$ – 0.14 mM up to 0.17 mM for MATSPHERES®–NH$_2$) of 1-(*S*)-phenylethanol before its complete inactivation. It needs to be stressed that the activity of each functionality under aerobic condition was similar among carries with the same functionality (see Tables 2 and 3).

In the absence of oxygen, a more active immobilized biocatalyst can be rapidly selected. Under anaerobic conditions, the enzyme operated for over 580 h (24 days), with residual activity still remaining for further 21 days, being able to convert several batches of substrate to achieve final 2.84 mM (56.5 µmol) (MATSPHERES®–OH) and 1.56 mM (31.2 µmol) (MATSPHERES®–NH$_2$) product concentrations (Fig. 5, Table 3). The average process activity determined as a change in product concentration from the start of the experiment until 580 h of reaction (the end of the linear rate of product formation) was 1.6 mU, 0.8 mU and 2.9 mU for the reactors with MATSPHERES®–OH, MATSPHERES®–NH$_2$ and free enzyme, respectively. For comparisons, the SBA preparations proved to be only slightly better than MATSPHERES® by 1.1 and 1.2 fold for SBA-OH and SBA-NH$_2$, respectively. Noteworthy, enzyme lifetime elongated almost 30 fold (from 20 h in aerobic conditions to over 580 h in anaerobic conditions).

Under the anaerobic conditions, the best results in terms of reaction efficiency were obtained with activation of –OH groups (for both of MATSPHERES® and SBA), which is in agreement with Tataruch et al., 2014 [27]. Moreover, enzymatic preparations on carriers with OH functionalities under aerobic conditions resulted in higher activity when compared to the –NH$_2$ supports. This could be the combined effect of two factors: i) presence of the DVS activator ii) lack of electron acceptor interaction with microsphere surface. In case of MATSPHERES®–NH$_2$, the electron acceptor depletion is probably caused by its reduction by reactive amine groups. This phenomenon may lead to serious decline in the activity of the enzyme immobilized on MATSPHERES®–NH$_2$.

Recently we have reported EbDH immobilisation on a cellulosic support [27], however, when flow reactors are concerned this type of carrier is inadequate due to its physical performance (gluing of grains thus clogging the column). MATSPHERES® however seems to be suitable for this type of applications and even outperforms standard SBA carrier used widely in immobilization studies with regards to their flow

Table 2
Catalytic characterization of immobilized and homogenous enzymes – EbDH and EL070.

Carrier	Activating agent	activity [mU] per mL (per mg) of carrier or homogenous enzyme*	Retained activity [%]	Bound protein** [mg ml^{-1}]	Enzyme loading (protein) [%]	Specific initial activity [mU mg^{-1}]
EbDH						
Homogenous EbDH	—	27.18	100	—	—	7.40
MATSPHERES® –OH	DVS	10.88 (0.041)	40	3.30	90	3.29
MATSPHERES® –NH$_2$	GA	9.60 (0.038)	35	3.23	88	2.98
SBA-OH	DVS	11.00 (0.106)	40	3.35	91	3.28
SBA-NH$_2$	GA	9.32 (0.063)	34	3.2	87	2.91
Lipase EL070						
Homogenous EL070	—	(274160.0)	100	—	—	274160.0
MATSPHERES® –OH	DVS	427920 (1620)	4.6	33.6	67	12735.7
MATSPHERES® –NH$_2$	GA	685450 (2740)	5.6	44.2	88	15507.9
SBA-OH	DVS	468738 (4521)	8.6	19.9	65	23554.7
SBA-NH$_2$	GA	279232 (1887)	3.7	27.3	89	10228.3

Where: DVS – divinyle sulphone, GA – glutaraldehyde.

* EbDH was obtained in liquid formulation from bacterial strain in our laboratory (see materials and methods), whereas lipase was purchased in powdered form. Therefore for homogenous EbDH the activity was given only "per ml", whereas in case of homogenous lipase "per mg".

** The amount of bound protein was calculated by subtracting the washed off protein quantity from this used for immobilization and recalculated per 1 ml of the carrier. Protein concentration for native EbDH (all supports) was 3.67 mg ml^{-1}, for lipase EL070 was 50 mg ml^{-1} and 30.2 mg ml^{-1} for MATSPHERES® and SBA, respectively.

Table 3
Synthesis of 1-(*S*)-phenylethanol under aerobic and anaerobic conditions.

Type of carrier	Final product concentration in the reactor [mM]	Reaction efficiency (based on max. product conc.) [%]	Final product concentration in the reactor [mM]	Reaction efficiency (based on max. product conc.) [%]
Reaction condition	anaerobic		aerobic	
Homogenous EbDH	4.90	100	0.40	100
MATSPHERES® –OH	2.84	58	0.16	40
MATSPHERES® –NH_2	1.56	32	0.17	43
SBA-OH	3.12	64	0.15	38
SBA-NH_2	2.17	44	0.14	35

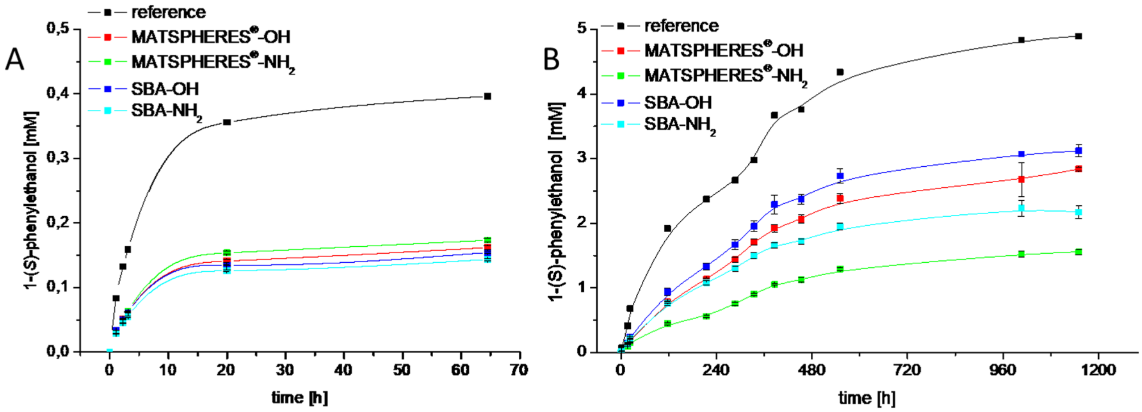

Fig. 5. Profiles of changes in product concentration in reaction catalysed by immobilized and native EbDH in aerobic (Panel A) and anaerobic (Panel B) atmosphere.

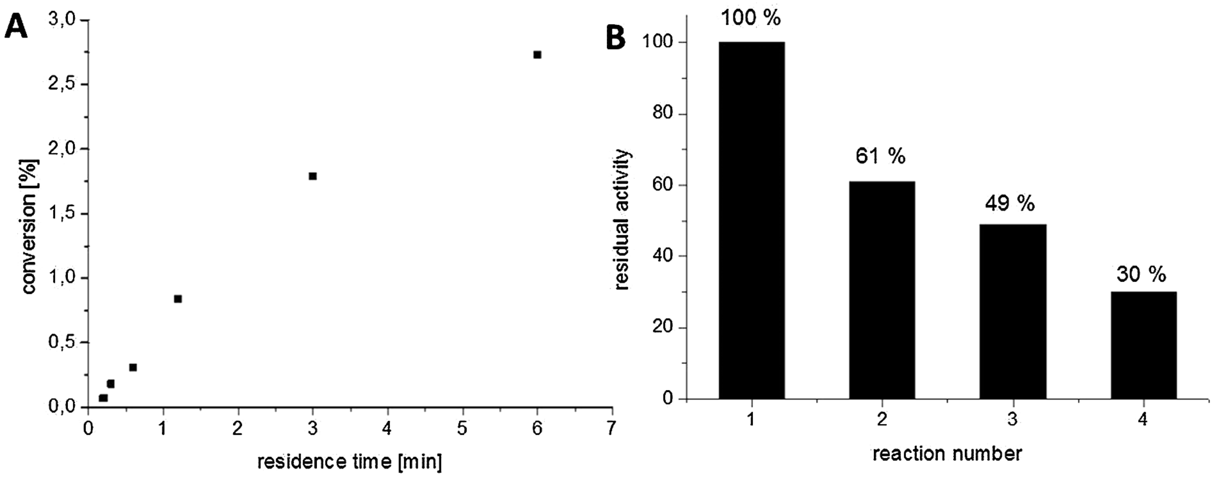

Fig. 6. Performance of immobilized EbDH in flow reactor. A – effect of residence time on acetophenone conversion; B – subsequent cycles in flow reactor.

characteristics (Fig. 2). We have tested the most promising enzyme formulation (EbDH immobilized on MATSPHERES®–OH) in the flow regime under anaerobic conditions by measuring the concentration of the product obtained at a specific flow rate. Fig. 6A shows the variation of product concentration with a change in flow rate in a single pass flow through the 0.5 ml of bed with EbDH immobilised on MATSPHERES®–OH in the reactor. In order to determine the reusability of the immobilised EbDH preparation (MATSPHERES®–OH), we performed a consecutive enzymatic test under a flow regime of 0.05 ml min^{-1}. 20 ml of the reaction medium with an initial concentration of 1.5 mM substrate was pumped through the silica bed in four reactor cycles under anaerobic conditions at a rate of 0.05 ml min^{-1} (6 h 20 min). After each charge, the column with the immobilized enzyme was washed with a fresh reaction buffer without substrate and stored under anaerobic atmosphere at 7 °C. The product concentration achieved after the first reaction reached 41 µM and was defined as 100% of activity. A drop in the activity was observed when consecutive cycle of enzymatic reaction were performed in the flow-through reaction (Fig. 6B). In the fourth charge, 30% of the residual activity was achieved in comparison to the first charge. 2.1 µmoles of the product was obtained in combined four charges in less than 27 h.

3.3.2. *EL070 lipase*

Mesoporous silica micro/nanoparticles, among other siliceous supports, also proved to be reliable carriers for lipases immobilisation [44–46]. However, little is known about the employment of hollow microspheres functionalised by DVS/GA with available –OH/–NH_2 surface active groups for lipase immobilisation and particularly for their use in sugar fatty acids ester (SFAE) synthesis. Therefore we have immobilised a commercial lipase on both types of carriers and tested the preparations for their usefulness in SFAE production. Based on tributyrin lipase activity assay and Bradford protein concentration assay it was determined that 0.127 mg of EL070 lipase was loaded on 1 mg of MATSPHERES®–OH giving finally 12.6U of enzyme activity, whereas 0.177 mg of lipase was loaded on 1 mg MATSPHERES®–NH_2 resulting in 15.5U of final enzyme activity (Table 2). Enzyme loading of the protein loading reached 67% for MATSPHERES®–OH and 88% for MATSPHERES®–NH_2. This may be due to the fact that silica inherently has hydroxyl groups on the surface, while by introducing additional NH_2 groups, the possibilities for the enzyme to be incorporated into the carrier are increased [37]. Immobilisation efficiency of EL070 on SBA was comparable to these obtained for spheres, namely 65% for SBA-OH and 88% for SBA-NH_2. Specific initial activity of SBA-OH preparation was 23.5 U mg^{-1} (1.8 fold greater than spheres), whereas for SBA-NH_2 we obtained 10.2 U mg^{-1} (0.6 fold lower than spheres). However, when specific activity values of the resulting preparations were compared with homogenous EL070 activity (274.2 U mg^{-1}), the efficiency of the whole process was only 4.6% and 5.7% for MATSPHERES®–OH and MATSPHERES®–NH_2, respectively; whereas for SBA-OH – 8.6% and SBA-NH_2 – 3.7%. These results suggest that during the immobilization procedure the enzyme was partially inaccessible. This may be due to covalent bond formation between functional groups of the protein and linking agents DVS or GA respectively, resulting in changes of the enzyme conformation that dislocates its active site [47–50].

Consecutively, we have employed free EL070 and its immobilised variants for biocatalytic synthesis of sugar esters in non-aqueous environments. Following protocols of others [23,41] the enzymatic reactions were performed in 2-methyl-2-butanol (2M2B) on its own (batch processes) or supplemented with 20% dimethyl sulfoxide (DMSO), in order to facilitate complete glucose solubilisation in the reaction medium during the flow type reactors. The free enzyme was found to poorly convert methyl nonanoate and glucose to its corresponding sugar ester in the batch tests (maximum of 13.5% glucose conversion, Table 4). However once immobilised on MATSPHERES®–OH and MATSPHERES®–NH_2, the lipase was able to produce glucose nonanoate in 2M2B with 88.5% and 98.4% efficiency, respectively (Table 4). Similar enhancement of SFAE synthesis was achieved, when lactose and palmitic acid were catalysed with immobilised *Candida antarctica* lipase B to form lactose palmitate [6]. For a benchmark comparison the EL070 immobilised on SBA supports and two other commercial immobilised lipase preparations were tested. We found that our two MATSPHERES® preparations of EL070 outperformed immobilised *Thermomyces lanuginosus* Amano lipase (TLIM) by 2.1–2.3 fold and *C. antarctica* lipase B (CalB) by 2.4–2.6 fold. When MATSPHERES®–OH where compared to their SBA counterparts conversions were at the same level. However, MATSPHERES®–NH_2 proved to be 1.2 fold more efficient than SBA-NH_2 (Table 4). It is also worth to notice that glucose nonanoate is not commercially available, therefore all conversion calculations were made taking into consideration the depletion of glucose in the reaction medium. However, the glucose nonanoate formation was confirmed by ESI-MS, ^1H NMR and IR measurements (see supplementary information).

Having in mind good performance of the hollow microspheres under different flow conditions, we set up flow type reactors in order to produce sugar esters in this mode. Being aware that sugar ester synthesis is a much slower process than EbDH biocatalysis, we decided to conduct experiments under the slowest flow possible under our experimental setup (0.02 ml min^{-1}). In order to avoid clogging of the reactor, we used 2M2B:DMSO (8:2, v/v) medium, as it allowed for a total glucose solubilisation. The column was packed with 250 µl of enzymatic bed and reactions were conducted 3 times at 55 °C and a flow rate of 0.05 ml min^{-1} (6 h 20 min). We have observed that MATSPHERES®–NH_2 enabled faster sugar conversion to glucose nonanoate after 1st and 2nd charges by 3.3 and 1.2 fold respectively when compared to MATSPHERES®–OH (final 88.3% versus 73.0% conversion, Table 5). The observed drop in the conversion for the 3rd round when the hollow microspheres were used can be explained by water sorption from the surrounding environment and ester hydrolysis (each round lasted for 6 h, set-up was not sealed and medium contained hygroscopic DMSO). Nevertheless, the investigated continuous flow system outperformed some of the previously reported SFAE continuous reactor setups [51–55].

Table 4

Synthesis of glucose nonanoate in 2-methyl-2-butanol in batch mode.

	Glucose after 24 h [mg ml^{-1}]	Conversion [%]
EL 070 homogenous	3.77	13.5
MATSPHERES® –OH	0.50	88.5
MATSPHERES® –NH_2	0.07	98.4
SBA-OH	0.50	89.1
SBA-NH_2	0.47	83.7
TLIM	2.53	42.1
CalB	2.72	37.6

Table 5

Performance of immobilised lipases in the flow reactors.

	Turnover	Conversion [%]			
		0	1	2	3
Type of support	MATSPHERES® –OH	0	12.9	73.0	51.1
	MATSPHERES® –NH_2	0	42.6	88.3	68.1

4. Conclusions

In the process of immobilization of ethylbenzene dehydrogenase on MATSPHERES® and SBA carriers with the same functionalization similar values of activity and bound protein were obtained, and thus the parameters characterizing the process of immobilization (enzyme loading, retained activity). At this stage there were no major differences between the spheres and the SBA carriers. Apart from the residual enzyme activity in the final phase of the reaction, it was shown that under anaerobic conditions the enzymatic preparations were able to operate for about 24 days (580 h), which corresponds to results reported already in the literature [27]. A prolonged operation (days) of enzymatic preparations in anaerobic conditions clearly indicated that for both supports without additional amino group (spheres or SBA) the immobilised enzyme operated more efficiently and differences in the final product concentrations or average process activities were very small.

Immobilisation of lipase enabled efficient production of sugar ester in either batch or flow-through regime. For immobilized lipase, the percentage of retained activity was significantly lower than in the case of EbDH (in the range of 3.7–8.6%). However, only the use of the immobilized formulation of this enzyme allowed effective glucose nonanoate synthesis. Within the silicas with amine groups, the retained activity for MATSPHERES® was 50% higher than for SBA, whereas this dependence was inverse for the NH_2-silica.

Silica carriers are widely used for immobilisation of a variety of enzymes. We have shown that hollow microspheres are also suitable for this type of applications. The MATSPHERES® are durable materials due to their openwork architecture that withstands harsh physical conditions (prolonged rotational mixing, freeze/thawing cycles, lyophilisation, etc.) revealing their broad applications in biocatalysis. Moreover, the hollow spheres enabled acceptable enzymes immobilisation, which are comparable to the gold standard in mesoporous silica immobilization – Santa Barbara Amorphous carriers.

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Conflict of interest

Mathilde Gosselind is an employee of Materium Innovations INC.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.02.038.

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